



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of :
Shinichiro ISOBE :
Serial No. 10/822,775 : Attn: BOX MISSING PARTS
Filed April 13, 2004 : Docket No. 2004_0569A
For: METHOD OF DETECTING BIOLOGICAL
MOLECULES, AND LABELING DYE AND
LABELING KIT USED FOR THE SAME

VERIFICATION OF ENGLISH TRANSLATION

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, Yasumasa HIRAI, declare:

that I am thoroughly familiar with both the Japanese and English languages;

that the attached document represents a true English translation of U.S. Patent Application Serial No. 10/822,775 filed April 13, 2004; and

that I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

July 22, 2004

DATE

Yasumasa Hirai

TRANSLATOR'S SIGNATURE



SPECIFICATION

TITLE OF THE INVENTION

METHOD OF DETECTING BIOLOGICAL MOLECULES, AND
5 LABELING DYE AND LABELING KIT USED FOR THE SAME

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to a method of detecting a biological
10 molecule such as nucleic acids, proteins, peptides, saccharides and the like
using a fluorescence dye, and a labeling dye and a labeling kit used for the
detection method.

Background Art

15 Recently, post genome researches have been intensively and world
widely done aiming for specific gene analysis technologies, gene therapies and
tailor made medical treatments. As for the gene analysis technology, a
method of detecting DNA using a DNA microarray, for example, is used.
According to this detection method, simultaneous analysis of expression,
20 functionality, mutation and the like of a plurality of genes can be conducted
simply and quickly.

In the detection method using a DNA microarray, DNA chips obtained
by spot-fixing many sequences (probe nucleic acids) of DNA or oligonucleotide
on a substrate made of glass, silicon or the like are used. By hybridization of
25 a probe nucleic acid fixed on a substrate with a labeled sample RNA or DNA

(target nucleic acid), a labeled nucleic acid having base sequence complimentary to that of the probe nucleic acid is selectively bound to the probe nucleic acid. After drying of the microarray, the fluorescence intensity of the labeled target nucleic acid is measured.

5 A fluorescence dye is widely used for labeling. High fluorescence intensity, emission even under dry conditions (solid conditions), water solubility, and the like are required for the fluorescence dye. As the fluorescence dye, for example, Cy3 and Cy5 are used (see, e.g., Science 283, 1 January 1999, pp. 83-87).

10

SUMMARY OF THE INVENTION

 However, although Cy3 and Cy5 manifest high fluorescence intensity and have a merit of emission even in solid state, they are very expensive, leading inevitably to a highly expensive detection method. Also, there is a
15 problem that the ratio of incorporation into a sample RNA or DNA is low and sufficient labeling of a sample RNA or DNA is impossible, resultantly, detection sensitivity is not sufficient. In contrast, fluorescence dye replacing Cy3 and Cy5 is not found to date.

 An object of the present invention is to solve the above-mentioned
20 problems and to provide a highly sensitive method of detecting a biological molecule at lower cost.

 The present inventors have found that an organic EL (electroluminescence)-dye used in an organic EL element manifests high fluorescence intensity when used as a label of a biological molecule, in a
25 process of searching for fluorescence dyes replacing Cy3 and Cy5, and achieved

the present invention.

Namely, the method of detecting a biological molecule according to one embodiment of the present invention is characterized by that it includes reacting a biological molecule sample with an organic EL-dye and measuring
5 the fluorescence of the biological molecule sample labeled with the organic EL-dye.

In the present invention, the biological molecule means a molecule species present in an organism, and includes those constituting the structure of an organism, those being concerned in production and conversion of energy,
10 those ruling bioinformation, and the like. Specifically included are nucleic acids, proteins, saccharides, lipids, peptides, nucleotides, metabolic intermediates and metabolic enzymes, hormones, and neurotransmitters, and the like.

Between an organic EL-dye and a biological molecule, an amide bond,
15 imide bond, urethane bond, ester bond, guanidine bond or thiourea bond can be formed. Prior to reaction with a biological molecule, any one reactive group selected from the group consisting of an isocyanate group, isothiocyanate group, epoxy group, halogenated alkyl group, triazine group, carbodiimide group and active ester carbonyl group can be introduced in the
20 above-mentioned organic EL-dye. Further, any one selected from the group consisting of nucleic acids, proteins, peptides and saccharides can be used as the biological molecule sample.

The method of detecting a biological molecule according to another embodiment of the present invention is characterized by that it includes
25 labeling of a biological molecule sample with a labeling dye comprising a

5-membered ring compound having a conjugate system and containing one or more hetero atom(s), selenium atom(s) or boron atom(s) and measurement of the fluorescence of the labeled biological molecule sample.

Also, a condensed poly-ring compound consisting of the
5 above-mentioned 5-membered ring compound and a 6-membered ring compound having a conjugate system may be used. Further, an azole derivative or imidazole derivative can be used as the 5-membered ring compound. Prior to reaction with the above-mentioned biological molecule, any one reactive group selected from the group consisting of an isocyanate
10 group, isothiocyanate group, epoxy group, halogenated alkyl group, triazine group, carbodiimide group and active ester carbonyl group can be introduced in an organic EL-dye.

The labeling dye according to another embodiment of the present invention is characterized by that it is a labeling dye used for detection of a
15 biological molecule by measurement of fluorescence, wherein the dye includes an organic EL-dye having a reactive group to bind to a biological molecule. As the reactive group, any one functional group selected from the group consisting of a carboxyl group, isocyanate group, isothiocyanate group, epoxy group, halogenated alkyl group, triazine group, carbodiimide group and active ester
20 carbonyl group can be used. As the organic EL-dye, a compound comprising a 5-membered ring compound having a conjugate system and containing one or more hetero atom(s), selenium atom(s) or boron atom(s) can be used. Further, a condensed poly-ring compound consisting of the above-mentioned 5-membered ring compound and a 6-membered ring compound having a
25 conjugate system may also be used. Furthermore, an azole derivative or

imidazole derivative may be used as the 5-membered ring compound.

The labeling kit for a biological molecule according to another embodiment of the present invention is characterized by that it includes an organic EL-dye for labeling a biological molecule. As the biological molecule,
5 any one selected from the group consisting of nucleic acids, proteins, peptides and saccharides can be used. As the reactive group for the biological molecule, any one functional group selected from the group consisting of a carboxyl group, isocyanate group, isothiocyanate group, epoxy group, halogenated alkyl group, triazine group, carbodiimide group and active ester carbonyl group can be used.
10 As the organic EL-dye, a compound comprising a 5-membered ring compound having a conjugate system and containing one or more hetero atom(s), selenium atom(s) or boron atom(s) can be used. Further, a condensed poly-ring compound consisting of the above-mentioned 5-membered ring compound and a 6-membered ring compound having a conjugate system may
15 also be used. Furthermore, an azole derivative or imidazole derivative may be used as the 5-membered ring compound.

For example, when used as a kit of DNA microarray, a nucleic acid is used as the biological molecule sample and a probe nucleic acid is fixed on a microarray, while the target nucleic acid sample is labeled by reaction with an
20 organic EL-dye, and the labeled target nucleic acid is spotted on the microarray and hybridization can be conducted under this conditions. Further, applying a binding property between avidin (streptavidin) and biotin, the avidin modified with this dye can be used as a biological assay kit for ELISA (enzyme-linked immunosorbent assay), Western blotting and the like.
25 It can also be used as a kit for protein array. A cell can also be stained, due to

efficient labeling of biological molecules such as saccharides and proteins.

According to the present invention, use of an organic EL-dye as a labeling dye for a biological molecule gives the following effects.

That is, an organic EL-dye shows high quantum yield in solid state
5 (including solid state and semi-solid state) and manifests high fluorescence intensity. Since an organic EL-dye is cheap as compared with Cy3 and Cy5, a biological molecule can be detected at lower cost. Further, an organic EL-dye reacts with a biological molecule almost quantitatively and shows high incorporation ratio, therefore, high detection sensitivity can be obtained.
10 Furthermore, use of said dye increases the degree of freedom of selectivity of fluorescence wavelength, and multiple fluorescence wavelengths of orange, yellow, green, blue and the like can be used. By this, it becomes possible to use two or more fluorescence dyes having large stokes shift (large difference between excited wavelength and fluorescence wavelength), consequently,
15 plural target nucleic acids contained in one sample can also be simultaneously detected. While Cy3 and Cy5 need to be kept in refrigerated state, an organic EL-dye is chemically stable and can be kept for a long time at ambient temperature, therefore, handling thereof is easy.

20 BRIEF EXPLANATION OF DRAWINGS

Fig. 1A shows one example of the HPLC profile of a labeled oligonucleotide in Example 1 of the present invention;

Fig. 1B shows one example of the UV spectrum of an intended labeled oligonucleotide in Example 1 of the present invention;

25 Fig. 2 shows one example of the TOF MS spectrum of a labeled

oligonucleotide in Example 1 of the present invention;

Fig. 3 shows one example of the emission pattern of a labeled oligonucleotide in Example 1 of the present invention, and (a), (b), (c) and (d) show results of 110 fmol, 10 fmol, 1 fmol and 0.5 fmol, respectively;

5 Fig. 4A shows one example of the HPLC profile of a labeled oligonucleotide in Example 2 of the present invention;

Fig. 4B shows one example of the UV spectrum of an intended labeled oligonucleotide in Example 2 of the present invention;

Fig. 5 shows one example of the emission pattern of a labeled oligonucleotide in Example 2 of the present invention, and (a), (b), (c), (d) and (e) show results of 500 fmol, 250 fmol, 100 fmol, 50 fmol and 10 fmol, respectively;

Fig. 6A shows one example of the HPLC profile of a labeled peptide in Example 3 of the present invention before purification;

15 Fig. 6B shows one example of the HPLC profile of a labeled peptide in Example 3 of the present invention after purification;

Fig. 7 shows one example of the TOF MS (Time of Flight Mass Spectrometry) spectrum of a labeled peptide in Example 3 of the present invention;

20 Fig. 8 shows one example of the emission pattern of a labeled peptide in Example 3 of the present invention, and (a), (b), (c), (d) and (e) show results of 10 fmol, 5 fmol, 1 fmol, 0.5 fmol and 0.1 fmol, respectively;

Fig. 9A shows one example of the TOF MS spectrum of a labeled protein in Example 4 of the present invention before labeling;

25 Fig. 9B shows one example of the TOF MS spectrum of a labeled

protein in Example 4 of the present invention after labeling; and

Fig. 10 shows one example of the emission pattern of a labeled protein in Example 4 of the present invention.

5 BEST MODES FOR CARRYING OUT THE INVENTION

Hereafter, embodiments of the present invention will be explained in detail.

The organic EL-dye used in the present invention is not particularly limited provided it is a dye sandwiched in solid state between a pair of anode
10 and cathode and capable of emitting by virtue of energy in recombination of a hole injected from an anode and an electron injected from a cathode. For example, poly-ring aromatic compounds such as tetraphenylbutadiene, perylene and the like, cyclopentadiene derivatives, distyrylpyrazine derivatives, acridone derivatives, quinacridone derivatives, stilbene
15 derivatives, phenothiazine derivatives, pyradinopyridine derivatives, azole derivatives, imidazole derivatives, carbazole derivatives, tetraphenylthiophene derivatives and the like can be used. Further, a dye having a carboxyl group in the molecule or into which a carboxyl group can be introduced is preferable. The reason for this is that a reactive group for bonding with a biological
20 molecule can be introduced easily as described below.

It is preferable that the organic EL-dye has a reactive group for bonding with a biological molecule sample (hereinafter, referred to target molecule) and the reactive group has a functional group capable of reacting with an amino group, imino group, thiol group, hydroxyl group, carboxyl group
25 or aldehyde group of the target molecule. It is preferable that an amide bond,

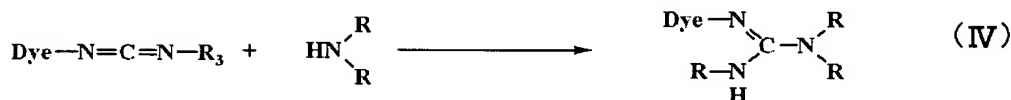
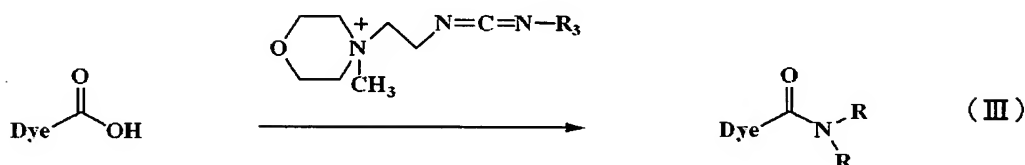
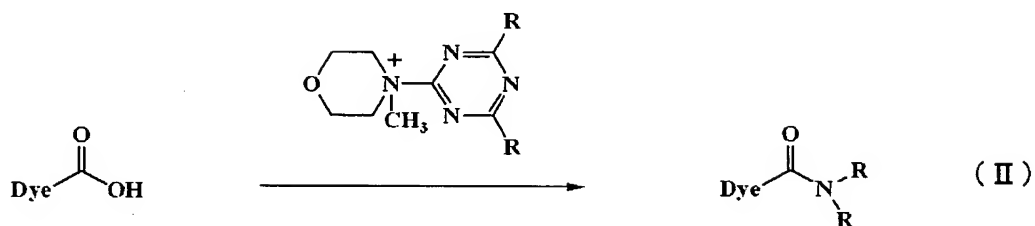
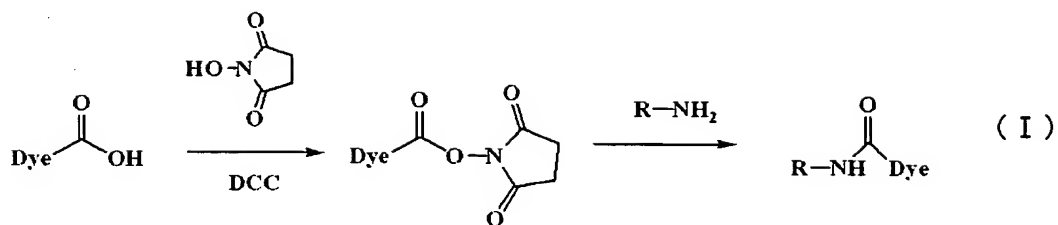
imide bond, urethane bond, ester bond, guanidine bond or thiourea bond is formed between an organic EL-dye and a biological molecule. As the functional group, for example, an isocyanate group, isothiocyanate group, epoxy group, halogenated sulfonyl group, acyl chloride group, halogenated alkyl group, glyoxal group, aldehyde group, triazine group, carbodiimide group and active ester carbonyl group and the like may be used. It is preferable that any one selected from the group consisting of an isocyanate group, isothiocyanate group, epoxy group, halogenated alkyl group, triazine group, carbodiimide group and active ester carbonyl group is used. The reason for this is that it can form an amide bond with an amino group in a target molecule and can directly bind to an imino group in a biological molecule. Further preferable is a triazine group, carbodiimide group or active ester carbonyl group. When these organic EL-dyes have a carboxyl group, an amino group and imino group present in a biological molecule can also be modified directly in the presence of a carbodiimide derivative and triazine derivative. Further, an organic EL-dye having a triazine group with an optional substituent or a carbodiimide group with an optional substituent can react directly with an imino group of guanine and thymine in DNA bases, therefore, introduction of a dye by a PCR (polymerase chain reaction) method is not necessary, and application thereof to mismatch detection and the like is possible.

For example, as the active ester carbonyl group, N-hydroxysuccinimide ester and maleimide ester can be used. By use of N-hydroxysuccinimide, an EL-dye and a target molecule can be bound by an amide bond via an N-hydroxysuccinimide ester using N,N'-dicyclohexylcarbodiimide (DCC) as a

condensing agent, as shown in formula I in the following Scheme 1. Further, as shown in formula II in the Scheme 1, a triazine derivative can also be used as the active ester carbonyl group. As the carbodiimide group, carbodiimide reagents such as DCC and 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide and
5 the like can be used. An EL-dye and a target molecule can be bound by an amide bond via a carbodiimide (formula III). Further, an EL-dye having previously a carbodiimide group or triazine group in the molecule can also be bound directly to an amino group and imino group in a biological molecule (formula IV).

10 Furthermore, excitation wavelength and emission wavelength can be changed by changing a substituent on an organic EL-dye, therefore, a plurality of samples can also be simultaneously detected by virtue of a plurality of colors.

Formula 1



Scheme 1

A reactive group can be bound with an amino residue modified at the
 5 end of oligo DNA when the target molecule is DNA, with amino residue in the
 case of a protein, an amino group of a polypeptide, for example, an amino
 residue of a polylysine derivative in the case of peptides, and with an amino
 group in a polysaccharide derivative skeleton in the case of a polysaccharide.

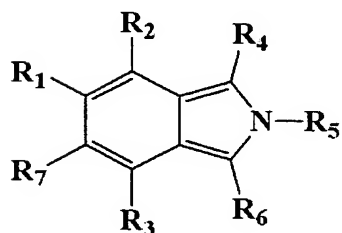
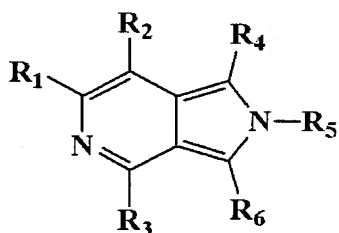
As the preferable organic EL-dye used in the detection method of the
 10 present invention, the compounds being comprised of a 5-membered ring

compound having a conjugate system and containing one or more hetero atom(s), selenium atom(s) or boron atom(s) are given. Further specifically, a mono-ring compound composed of a 5-membered ring compound having a conjugate system, and a condensed poly-ring compounds consisting of
5 6-membered ring compound having a conjugate system and the 5-membered ring compound are given. The reason for this is that they have large quantum yield and show intense fluorescence even in solid state.

Specific examples of the condensed poly-ring compound are explained below.

10 (Mono-azole derivative 1)

Formula 2

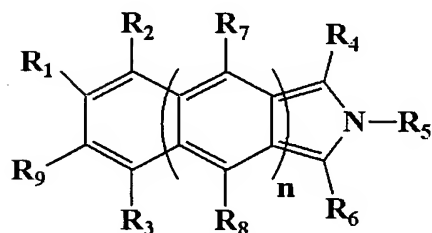
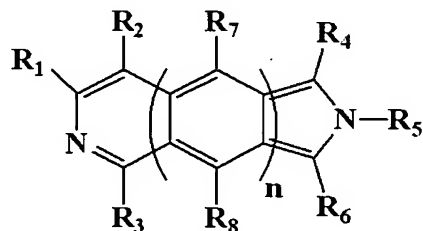


(wherein, R₁, R₂, R₃, R₄, R₆ and R₇ represent each independently an aromatic hydrocarbon group, hydrocarbon group, heterocyclic group or aromatic group containing a hetero atom in the ring, optionally having a substituent such as a
15 hydrogen atom, halogen atom, hydroxyl group, cyano group or sulfonyl group

and the like. R_1, R_2, R_3, R_4, R_6 and R_7 may be the same or different.) These are the same also in the following general formulae unless otherwise stated.

(Monoazole derivative 2)

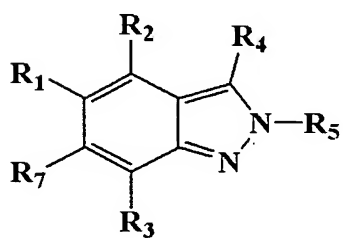
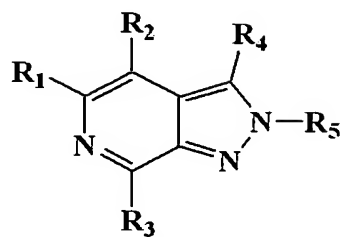
Formula 3



- 5 (wherein, R_8 and R_9 represent each an aromatic hydrocarbon group, hydrocarbon group, heterocyclic group or aromatic group containing a hetero atom in the ring, optionally having a substituent such as a hydrogen atom, halogen atom, hydroxyl group, cyano group or sulfonyl group and the like. R_8 and R_9 may be the same or different.) These are the same also in the
- 10 following general formulae unless otherwise stated. (wherein, n represents an integer of 1 or more, preferably of 1 to 5.) This is the same also in the following general formulae.

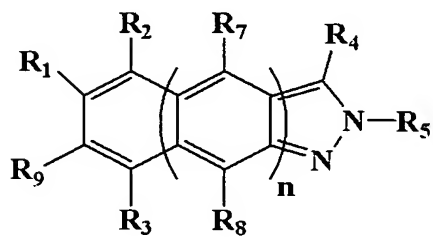
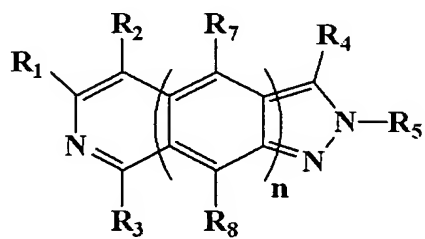
(Diazole derivative 1)

Formula 4



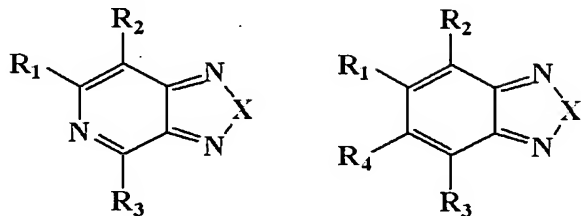
(Diazole derivative 2)

Formula 5



(Diazole derivative 3)

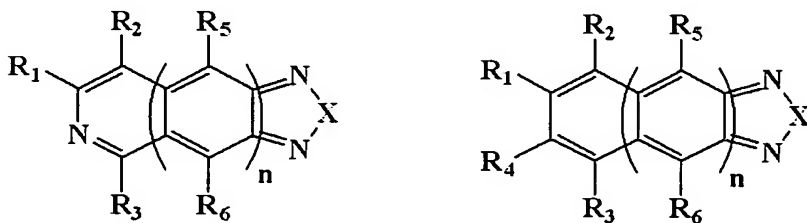
Formula 6



(wherein, R_1 , R_2 , R_3 and R_4 represent each independently an aromatic hydrocarbon group, hydrocarbon group, heterocyclic group or aromatic group containing a hetero atom in the ring, optionally having a substituent such as a hydrogen atom, halogen atom, hydroxyl group, cyano group or sulfonyl group and the like. R_1 , R_2 , R_3 , R_4 , R_6 and R_7 may be the same or different. As R_2 and R_3 , aromatic hydrocarbon groups optionally having a substituent are preferable, and as this substituent, alkyl groups and alkoxy groups having 1 to 4 carbon atoms, or a bromine atom are preferable. Further, as the alkyl group, a methyl group, and as the alkoxy group, a methoxy group, are preferably used, respectively. X represents a nitrogen atom, sulfur atom, oxygen atom, selenium atom or boron atom, optionally having a substituent.) This is the same also in the following general formulae unless otherwise stated.

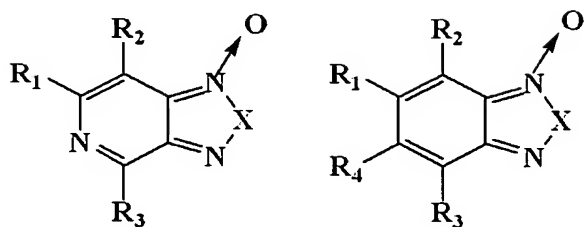
(Diazole derivative 4)

Formula 7



(Diazole derivative 5)

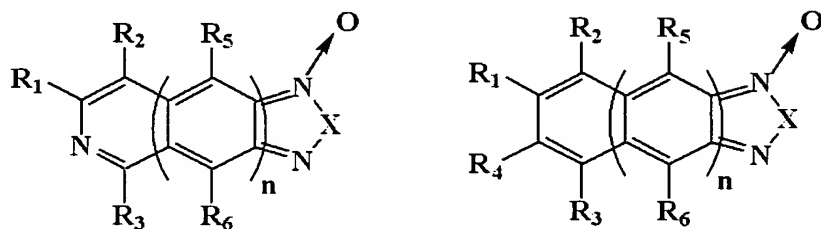
Formula 8



(wherein, $N \rightarrow O$ represents a state in which a nitrogen atom is coordinate-bonded to an oxygen atom.)

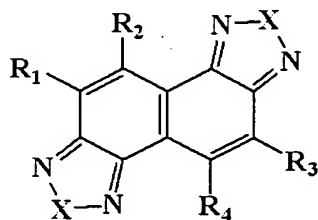
5 (Diazole derivative 6)

Formula 9



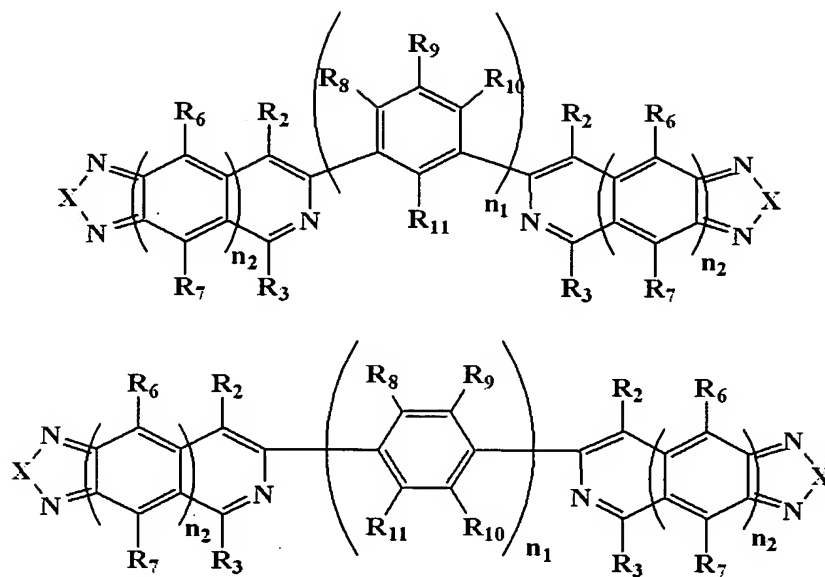
(Diazole derivative 7)

Formula 10

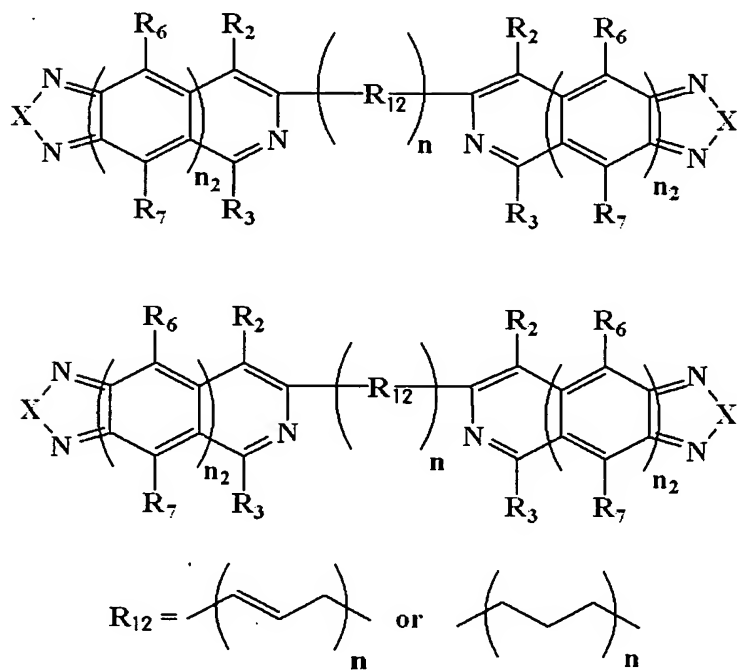


(Diazole derivative 8)

Formula 11-1



Formula 11-2

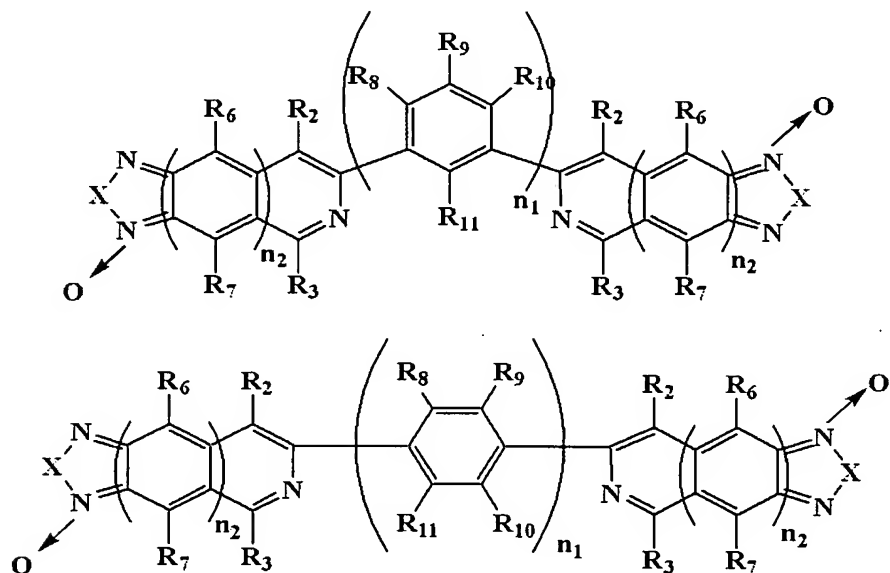


(wherein, R_{10} and R_{11} represent each an aromatic hydrocarbon group, hydrocarbon group, heterocyclic group or aromatic group containing a hetero atom in the ring, optionally having a substituent such as a hydrogen atom, halogen atom, hydroxyl group, cyano group or sulfonyl group and the like.

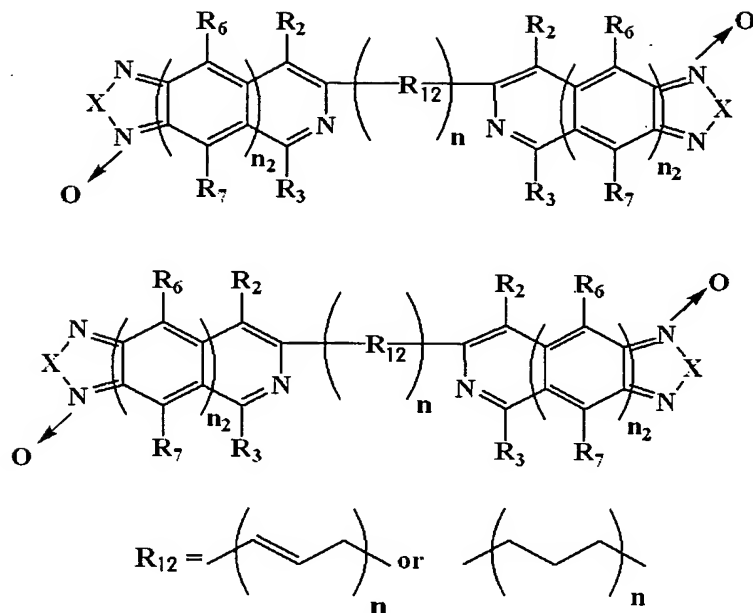
5 R_{10} and R_{11} may be the same or different. R_{12} is an olefin group or paraffin group optionally having a substituent, and n represents an integer of 1 to 3, preferably 1.) These are the same in the following formulae unless otherwise stated.

(Diazole derivative 9)

10 Formula 12-1

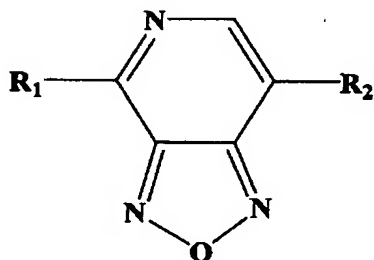


Formula 12-2



The above-mentioned diazole derivatives are not particularly limited, but an oxadiazolopyridine derivative of the following general formula can be suitably used.

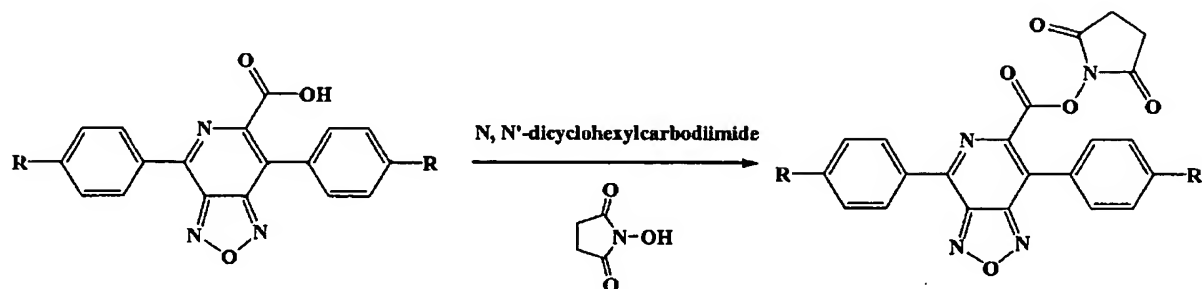
Formula 13



Regarding the oxazolopyridine derivative, its carboxylic acid derivative is synthesized, then, it is derived into an active ester containing N-hydroxysuccinimide ester using N,N'-dicyclohexylcarbodiimide (DCC) as a

condensing agent, for example, according to a reaction shown in the following Scheme 2, and the resulting derivative is used.

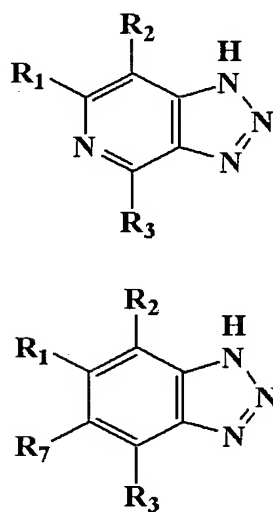
Formula 14



5 Scheme 2

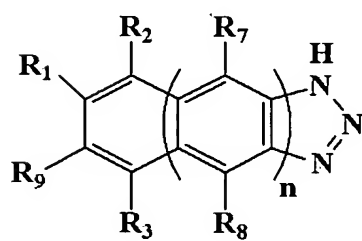
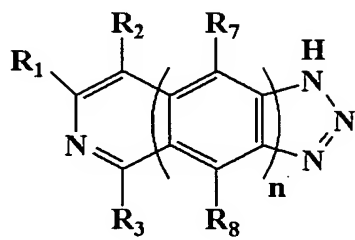
(Triazole derivative 1)

Formula 15



(Triazole derivative 2)

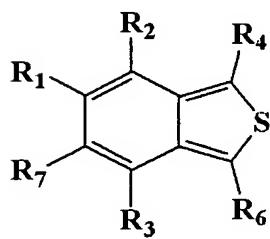
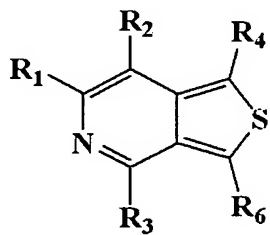
Formula 16



As the 5-membered ring compound, the following derivatives containing a thiophene group can also be used.

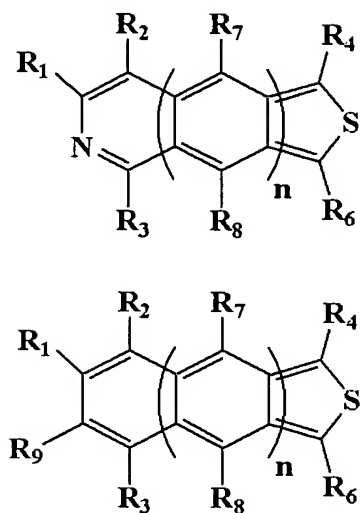
5 (Thiophene derivative 1)

Formula 17



(Thiophene derivative 2)

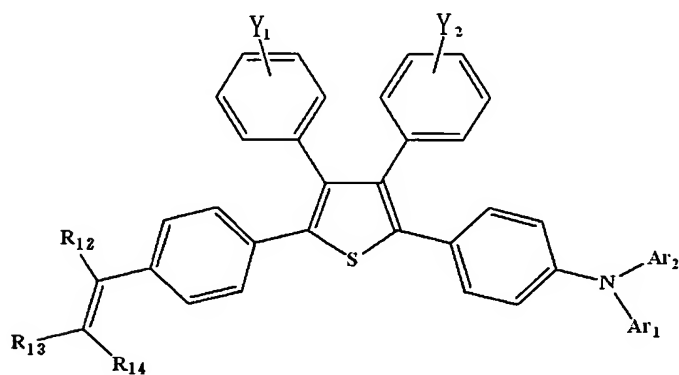
Formula 18



(Thiophene derivative 3)

In the case of a thiophene derivative, a 2,3,4,5-tetraphenylthiophene derivative which is a non-condensed type compound and represented by the following general formula can also be used.

Formula 19



(wherein, R₁₂, R₁₃ and R₁₄ represent each independently a hydrogen atom,

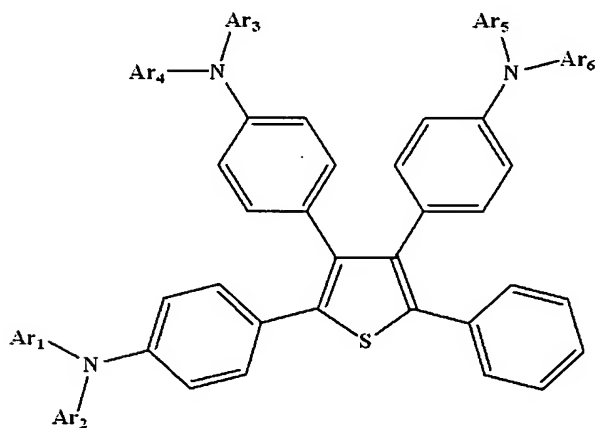
linear, branched or cyclic-alkyl group, substituted or unsubstituted aryl group, or substituted or unsubstituted aralkyl group, Ar₁ and Ar₂ represent a substituted or unsubstituted aryl group, further, Ar₁ and Ar₂ may form a nitrogen-containing heterocyclic ring together with a bonded nitrogen atom.

5 Y₁ and Y₂ represent a hydrogen atom, halogen atom, linear, branched or cyclic-alkyl group, linear, branched or cyclic-alkoxy group, substituted or unsubstituted aryl group, substituted or unsubstituted aralkyl group, or substituted or unsubstituted amino group.)

(Thiophene derivative 4)

10 A 2,3,4,5-tetraphenylthiophene derivative of the following general formula can also be used.

Formula 20



(wherein, Ar₁ to Ar₆ represent each independently a substituted or unsubstituted aryl group, further, Ar₁ and Ar₂, Ar₃ and Ar₄, and Ar₅ and Ar₆ may form a nitrogen-containing heterocyclic ring together with a bonded nitrogen atom.)

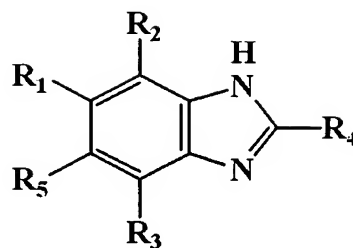
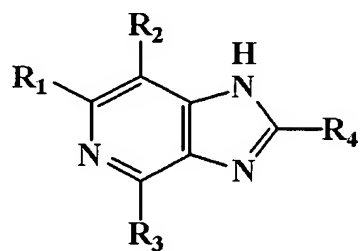
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Further, an imidazole can also be used as the 5-membered ring compound, for example, imidazole derivatives of the following general

formulae.

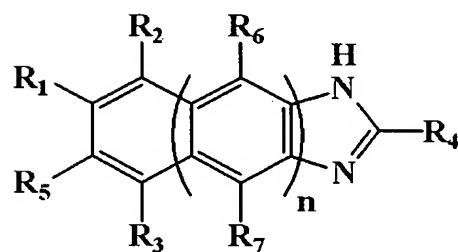
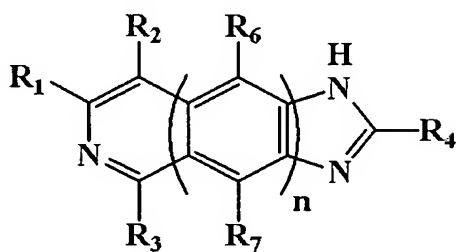
(Imidazole derivative 1)

Formula 21



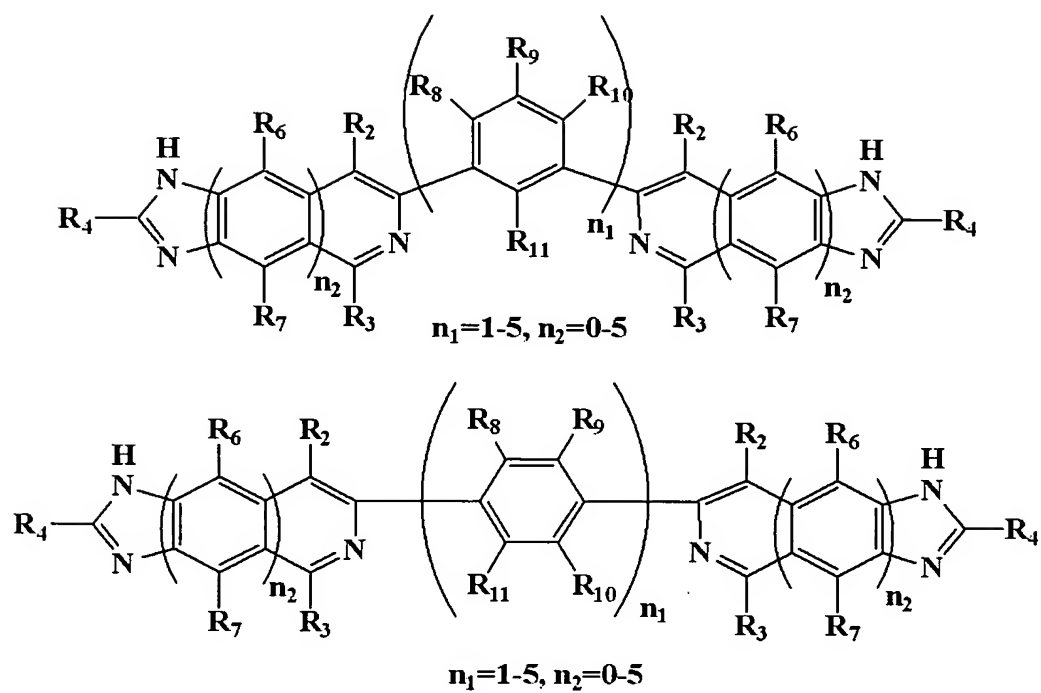
5 (Imidazole derivative 2)

Formula 22

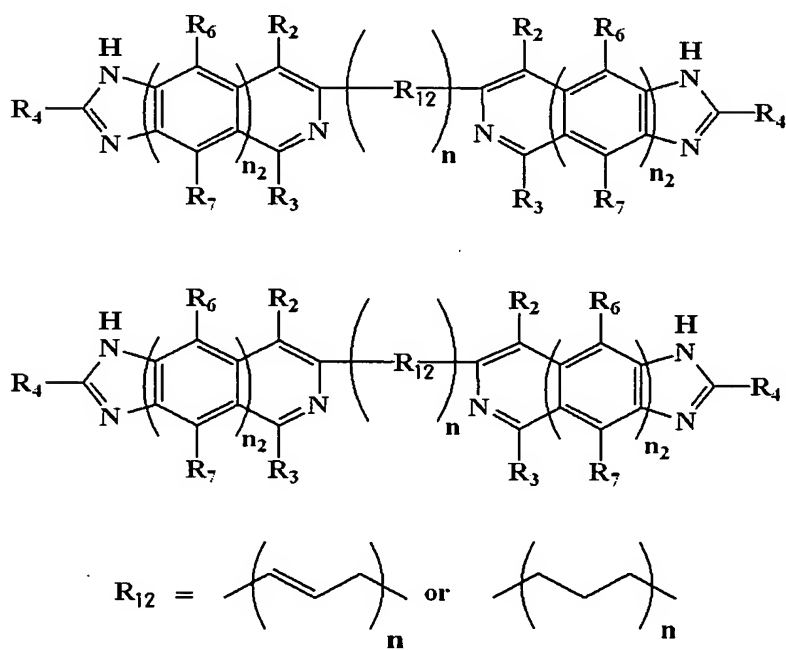


(Imidazole derivative 3)

Formula 23-1



Formula 23-2

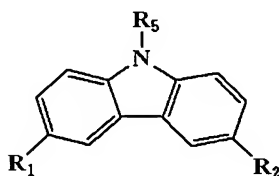


(wherein, in the imidazole skeleton, a plurality of units may be bonded to any position of center benzene rings R₈, R₉, R₁₀ and R₁₁. R₁₂ is an olefin group or paraffin group optionally having a substituent, and n represents an integer of 1 to 3, preferably 1.).

5 (Carbazole derivative)

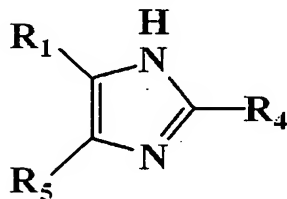
A carbazole derivative of the following general formula can also be used.

Formula 24



10 Further, a 5-membered ring compound which is a mono-ring compound having a conjugate system and containing one or more hetero atom(s), selenium atom(s) or boron atom(s) can also be used. Though not particularly limited, azole derivatives of the following general formula, for example, can also be used.

15 Formula 25



(wherein, R₁, R₄ and R₅ represent each independently an aromatic hydrocarbon group, hydrocarbon group, hetero-cyclic group or aromatic group containing a hetero atom in the ring, optionally having a substituent such as a

hydrogen atom, halogen atom, hydroxyl group, cyano group or sulfonyl group and the like. R₁, R₄ and R₅ may be the same or different.)

The detection method of the present invention can be applied to any detection method provided it is a method of measuring the fluorescence of a
5 labeled biological molecule in solid or semi-solid state. For example, when used for gene analysis using a DNA microarray, it can be conducted according to the following procedure.

In the case of investigation of a gene expression, those prepared by amplifying cDNA and the like by a PCR method using a cDNA library, genome
10 library or whole genome as a template can be used as the probe nucleic acid to be fixed on a substrate. In the case of investigating of a gene mutation and the like, various oligonucleotides corresponding to the mutation and the like synthesized based on a known sequence as a standard can be used.

Fixation of a probe nucleic acid onto a substrate can be conducted by a
15 suitable method selected depending on the kind of a nucleic acid and the kind of a substrate. For example, a method can also be used in which a probe nucleic acid is electrostatically-bonded to a substrate surface-treated with a cation of polylysine and the like by use of the charge of DNA.

On the other hand, a labeled target nucleic acid with an organic
20 EL-dye is prepared by mixing a target nucleic acid and an organic EL-dye and reacting together. The reaction temperature is preferably from room temperature to 60°C and the reaction time is preferably from 2 to 48 hours.

Then, the labeled target nucleic acid is spotted on a substrate, and hybridization thereof is conducted. Hybridization is preferably conducted at
25 from room temperature to 70°C for 2 to 48 hours. By hybridization, a target

nucleic acid having a base sequence complimentary to a probe nucleic acid is selectively bonded to a probe nucleic acid. Thereafter, the substrate is washed and dried at room temperature.

Then, the fluorescence intensity of the surface of the dried substrate is
5 measured by a fluorescence laser scanner method. By fluorescence intensity, the level of gene expression can be monitored.

Regarding the above-mentioned hybridization, a method of fixing a probe nucleic acid onto a substrate has been explained, however, a method can also be used in which a target nucleic acid labeled previously with an organic
10 EL-dye is fixed on a substrate and a probe nucleic acid is spotted on a substrate.

The labeling kit of the present invention contains an organic EL-dye or derivative thereof for labeling a biological molecule, and if necessary, it can contain reagents, enzymes, solvents and the like, for reacting a dye with the
15 object biological molecule. The object biological molecules include a nucleic acid, protein, peptide or saccharide. The organic EL-dye is preferably a derivative having a functional group reacting with an amino group of a biological molecule. Examples of the functional group preferably include any one selected from an isocyanate group, isothiocyanate group, epoxy group,
20 halogenated alkyl group, triazine group, carbodiimide group and active ester carbonyl group. Further preferably, an active ester containing a triazine group, carbodiimide group or active ester carbonyl group is contained as a derivative of an organic EL-dye.

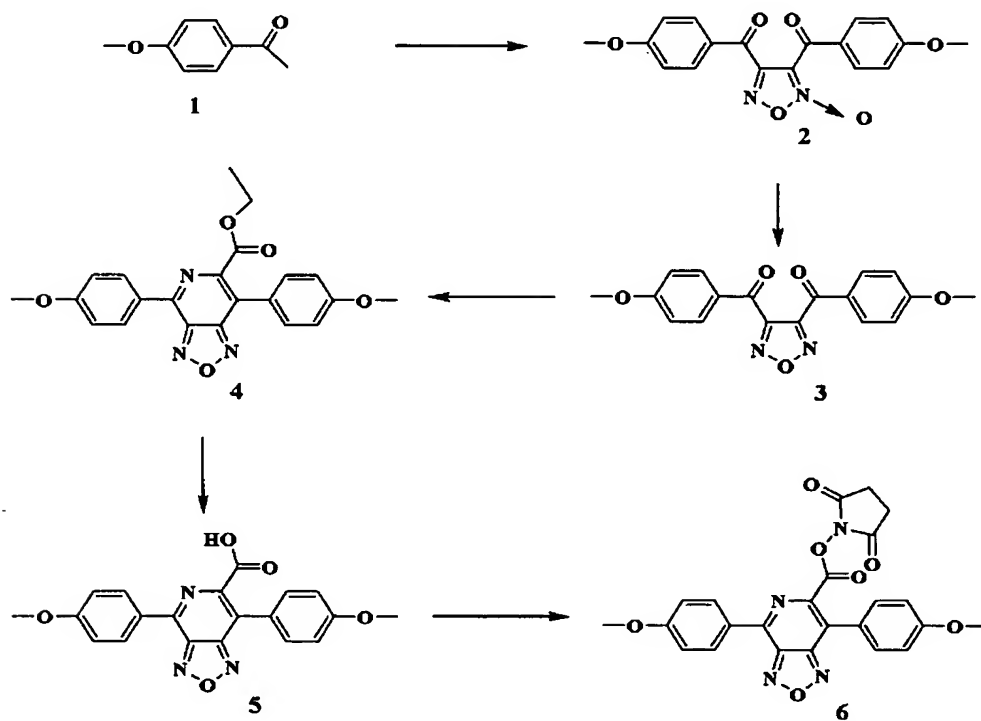
The present invention will be further specifically explained in more
25 detail in the following examples.

Synthesis Example 1

A 1,2,5-oxadiazolo-[3,4-c]pyridine derivative was used as the organic EL-dye.

The scheme for synthesis of an active ester of a 1,2,5-oxadiazolo-[3,4-c]pyridine (hereinafter, abbreviated as EL-OSu) will be shown below.

Formula 26



Scheme 3

(1) Synthesis of diketone derivative (2)

In a 500 mL three-necked flask, 37.5 g (0.25 mol) of 4-methoxyacetophenone (1) and 0.15 g of sodium nitrite were dissolved in 100

mL of acetic acid. On a water bath, a solution prepared by dissolving 100 mL of HNO_3 in 100 mL of acetic acid was added dropwise over 2 hours. Then, the mixture was stirred at room temperature for 2 days. The reaction mixture was slowly added into 500 mL of water to cause precipitation. The precipitate
5 was filtrated and dissolved in chloroform. The chloroform phase was washed with saturated sodium bicarbonate water, and washed twice with a 10% NaCl aqueous solution. After dehydration over MgSO_4 , chloroform was distilled off under reduced pressure to obtain 34.5 g (yield: 78%) of oxadiazole-N-oxide (2).

(2) Synthesis of diketone derivative (3)

10 In a 500 mL three-necked flask, 17.7 g (0.05 mol) of oxadiazole-N-oxide (2) was dissolved in 400 mL of acetonitrile. Into this was added 12.0 g of Zn, 7 mL of AcOH and 20 mL of Ac_2O . On a water bath, the resulted mixture was cooled so that the reaction temperature did not exceed 30°C . The mixture was stirred for 12 hours to terminate the reaction. The reaction mixture was
15 filtrated to remove insoluble materials. Acetonitrile was distilled off under reduced pressure to obtain a residue. The residue was recrystallized from chloroform to obtain 10.2 g (yield: 60%) of oxadiazole-N-oxide (3).

(3) Synthesis of oxadiazolopyridine ethyl ester (4)

In a 500 mL three-necked flask, 15.6 g (0.046 mol) of
20 oxadiazole-N-oxide (3) was dissolved in 300 mL of butanol. Into this was added 32.0 g (0.23 mol) of a glycine ethyl ester hydrochloride. The mixture was heated to reflux for 24 hours. Butanol was distilled off under reduced pressure to obtain a residue. The residue was dissolved in 200 mL of chloroform, and washed with 10% HCl, saturated NaHCO_3 and 10% NaCl.
25 This was dried over MgSO_4 and the solvent was distilled off. The resulted

residue was recrystallized from chloroform to obtain 13.0 g (yield: 70%) of oxadiazolopyridine ethyl ester (4).

(4) Hydrolysis of oxadiazolopyridine ethyl ester (4)

In a 500 mL three-necked flask, 3.0 g (0.007 mol) of oxadiazolopyridine ethyl ester (4) was dissolved in 200 mL of ethanol. To this was added 0.62 g (0.01 mol) of KOH. After heating to reflux for 5 hours, the reaction mixture was added to 200 mL of water. Into this aqueous solution, concentrated hydrochloric acid was added dropwise to adjust pH to 1 to obtain a precipitate. The precipitate was filtrated and dissolved in chloroform. The chloroform phase was washed with a 10% NaHCO₃ aqueous solution and water. Chloroform was distilled off to obtain a residue. The residue was recrystallized from water-ethanol (1:1) to obtain 2.1 g (yield: 81%) of oxadiazolopyridinecarboxylic acid (5)

Synthesis of active ester (6)

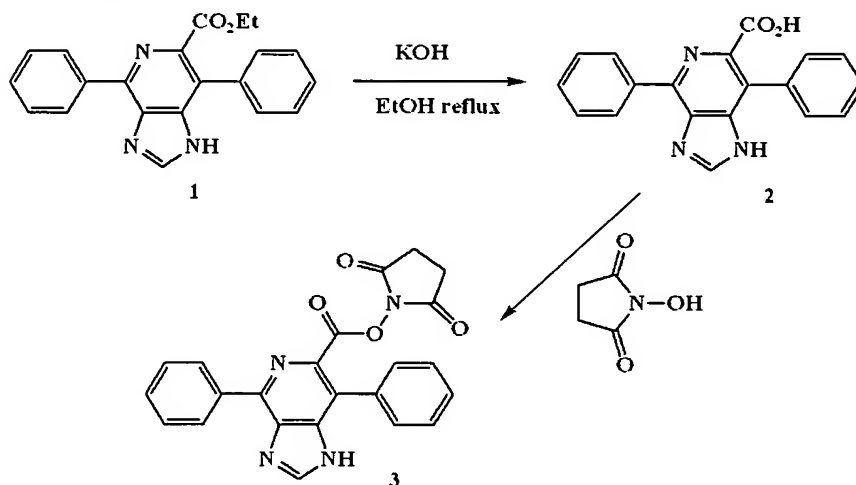
In a 50 mL three-necked flask, 1.0 g (0.0026 mol) of oxadiazolopyridinecarboxylic acid (5) and 0.30 g (0.0026 mol) of N-hydroxysuccinimide were dissolved in 20 mL of DMF. Into this, 0.54 g (0.0026 mol) of N,N'-dicyclohexylcarbodiimide was added dropwise over 30 minutes. After dropping, the mixture was stirred for 30 hours at room temperature. Under reduced pressure, DMF was distilled off. The residue was isolated and purified by silica gel column chromatography (chloroform) to obtain 0.76 g (yield: 62%) of an oxadiazolopyridine active ester (6).

Synthesis Example 2

An imidazolopyridine ethyl ester derivative was used as a organic

EL-dye. The scheme for synthesis of an active ester of a imidazolopyridine ethyl ester (hereinafter, abbreviated as im-EL-OSu) will be shown below.

Formula 27



5 Scheme 4

(1) Hydrolysis of imidazolopyridine ethyl ester (1)

In a 500 mL three-necked flask, 0.5 g (1.5 mmol) of an ester 1 was dissolved in 50 mL of ethanol. To this was added 0.12 g (2.1 mol) of KOH. After heating to reflux for 5 hours, the reaction mixture was added to 50 mL of water. Into this aqueous solution, concentrated hydrochloric acid was added dropwise to adjust pH to 1 to obtain a precipitate. The precipitate was filtrated and dissolved in chloroform. The chloroform phase was washed with a 10% NaHCO₃ aqueous solution and water. Chloroform was distilled off to obtain a residue. The residue was recrystallized from water to obtain 0.3 g (yield: 63%) of a carboxylic acid 2.

(2) Synthesis of active ester (3)

In a 50 mL three-necked flask, 0.2 g (0.6 mmol) of a carboxylic acid

derivative 2 and 0.07 g (0.6 mmol) of N-hydroxysuccinimide were dissolved in 10 mL of DMF. Into this, 0.12 g (0.6 mmol) of N,N'-dicyclohexylcarbodiimide was added dropwise over 30 minutes. After dropping, the mixture was stirred for 30 hours at room temperature. Under reduced pressure, DMF was
 5 distilled off. The residue was isolated and purified by silica gel column chromatography (chloroform) to obtain 0.14 g (yield: 55%) of an active ester 3.

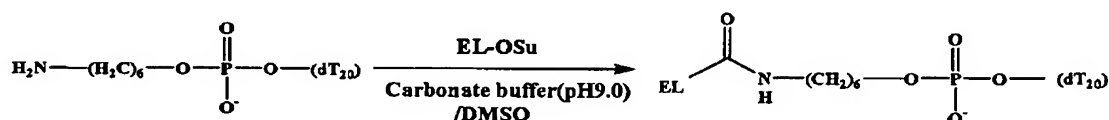
Example 1

<Labeling of oligonucleotide with dye, and detection thereof (1)>

10 1. Labeling of oligonucleotide with dye

Labeling of an oligonucleotide with a dye was conducted according to the following Scheme 4.

Formula 28



15 Scheme 4.

(Experimental Procedure)

Into 40 µl of Na₂CO₃/NaHCO₃ buffer (pH 9.0) containing H₂N-dT₂O (40 mmol) was added 12 µl of an anhydrous DMSO solution containing 5.0 µmol (2.4 mg) of an active ester of an organic EL-dye and the mixture was shaken at
 20 room temperature for 6 hours. After shaking, 0.1 M TEAA (triethylamine acetic acid) buffer (pH 7.0) was added so as to give the total volume of 1 ml, and components derived from the oligonucleotide were separated using

NAP-10 column (Pharmacia Sephadex G-25). In this operation, the NAP-10 column had been equilibrated previously with 15 ml of 0.1 M TEAA buffer before use. The sample solution of which total volume had been adjusted to 1 ml was applied into in a column. After elution of 1 ml of the solution, 0.1 M TEAA buffer was charged in a volume of 1.5 ml. Immediately after this, 1.5 ml of the eluted solution was separated. The resulted solution was freeze-dried over night, and 20 μ l of sterile distilled water was added and analyzed by reverse phase HPLC. The solution injected into HPLC was previously diluted to 1/40 and analyzed.

10 (HPLC measurement conditions)

Column: Lichrospher RP-18 (Cica-MERCK)

Flow rate: 1 ml/min

Detection wavelength: 260 nm

Sample injection solvent: ultra-pure water

15 Eluent A: 0.1 M TEAA buffer (pH 7.0), 10% CH₃CN solution

Eluent B: 0.1 M TEAA buffer (pH 7.0), 40% CH₃CN solution

Table 1 Gradient conditions of HPLC measurement

	0	30	35	40 (min)
A	100	0	0	100 (%)
B	0	100	100	0 (%)

The HPLC profile of the labeled oligonucleotide and the UV spectrum of the intended material are shown in Fig. 1A and Fig. 1B, respectively. As a result of HPLC, a peak around RT = 30 min was confirmed to be the intended material, and preparative HPLC was conducted. Identification of the resulted

intended material was conducted by MALDI (Matrix Assisted Laser Desorption/Ionization) TOF MS. The result is shown in Fig. 2. The reaction ratio was calculated from the peak area in HPLC chromatogram to find a ratio of about 90%, namely, the active ester (6) of an EL-dye was reacted almost
5 quantitatively with oligo DNA.

2. Detection of labeled oligonucleotide

Next, solutions of different concentration of the labeled oligonucleotide were prepared as shown in the following Table 2. Then, 1 nL of the solution was spotted on a glass substrate (5 × 5). After spotting, the glass substrate
10 was dried.

Table 2

Solution concentration (μM)	Relative concentration of labeled oligonucleotide (fmol)
110	110
11	11
1	1
0.5	0.5

Next, the detection limit thereof was investigated by a fluorescence scanner. The results are shown in Table 3. Here, (a), (b), (c) and (d) show
15 results of 110 fmol, 10 fmol, 1 fmol and 0.5 fmol, respectively.

Here, as the detection instrument, BIO-RAD molecule imager FX Pro was used. The laser wavelength was 488 nm and the scan interval was 50 nm.

(Result)

20 The excitation light used in this detection is laser light of 488 nm and the excitation wavelength of the fluorescence dye is 438 nm. Irrespective of

this, the detection limit of the relative concentration of the labeled oligonucleotide was 0.5 fmol (500 amol), and therefore, detection in high sensitivity was possible. The reaction with DNA was almost quantitative, and the reaction time could be reduced from conventional times of about 24 hours to about 6 hours. Further, this EL-dye was stable, and even when re-measurement was conducted using an EL-dye preserved at room temperature for 15 days, the equivalent results were obtained.

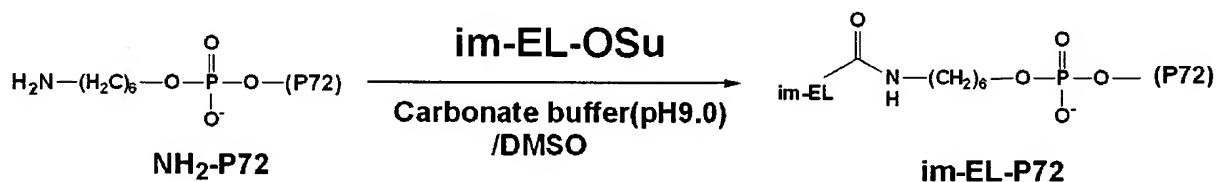
Example 2

<Labeling of oligonucleotide with dye, and detection thereof (2)>

1. Labeling of oligonucleotide with dye

Labeling of an oligonucleotide with a dye was conducted according to the following Scheme 4. The labeling conditions are the same as that for Example 1. The addition reaction of an imidazole derivative progressed quickly and almost quantitatively.

Formula 29



Scheme 5.

(HPLC measurement conditions)

Column: Lichrospher RP-18 (Cica-MERCK)

Flow rate: 1 ml/min

Detection wavelength: 260 nm

Sample injection solvent: ultra-pure water

Eluent A: 0.1 M TEAA buffer (pH 7.0), 10% CH₃CN solution

Eluent B: 0.1 M TEAA buffer (pH 7.0), 40% CH₃CN solution

5 The gradient conditions of HPLC measurement are the same as that of Example 1. The HPLC profile of the labeled oligonucleotide and the UV spectrum of the intended material are shown in Fig. 4A and Fig. 4B, respectively. As a result of HPLC, a peak around RT = 25 min was confirmed to be the intended material, and preparative HPLC was conducted.

10 Next, the detection limit thereof was investigated by fluorescence scanner in the same manner as that of the example. The results are shown in Fig. 5. Here, (a), (b), (c), (d) and (e) show emission patterns of 500 fmol, 250 fmol, 100 fmol, 50 fmol and 10 fmol, respectively.

(Result)

15 The detection limit of the relative concentration of the labeled oligonucleotide was 10 fmol, and therefore, detection in high sensitivity was possible. The reaction of the oligonucleotide and EL-dye was almost quantitative.

20 Example 3

<Labeling and detection of peptides>

1. Synthesis of Ac-Lys(EL)-Lys-Lys-Lys(Acr)-Lys-Lys-Lys(Acr)-Lys-Lys-NH₂

(1) Synthesis of

Ac-Lys(Mtt)-(Lys(Boc))₂-Lys-(Acr)-(Lys(Boc))₂-Lys(Acr)-(Lys(Boc))₂-Resin

25 (where, Lys denotes lysine, Mtt denotes 4-methyltrityl, Boc denotes

tert-butyloxycarbonyl, and Acr denotes acridinyl, respectively)

(Experimental Procedure)

Into a reaction vessel, 0.15 g (0.61 mmol/g) of Fmoc
(9-fluorenylmethyloxycarbonyl) - NH- SAL (super acid labile) Resin was
5 charged, and 0.26 g of Fmoc-Lys(Acr)-OH was added in each of cartridges 3, 6,
0.18 g of Fmoc-Lys(Boc)-OH was added in each of cartridges 1, 2, 4, 5, 7 and 8,
and 0.23 g of Fmoc-Lys(Mtt)-OH was added in a cartridges 9. Subsequently,
synthesis was conducted using 431A peptide synthesizer of Applied Biosystems.
The standard Fmoc method was followed, and the N-terminal was acetylated.
10 A yellow solid peptide resin was obtained. The yield was 0.30 g.

(2) Deprotection of Mtt group of

Ac-Lys(Mtt)-(Lys(Boc))₂-Lys-(Acr)-(Lys(Boc))₂-Lys(Acr)-(Lys(Boc))₂-Resin, and
modification by EL, cleavage from resin, and deprotection of side chain

(Experimental Procedure)

15 i) Deprotection of Mtt group

Into a screw tube, 0.30 g of the peptide resin synthesized in 1 was
charged, and to this was added excess amount of dichloromethane (DCM) and
swollen over 30 minutes, then excess DCM was removed by a nitrogen gas.
Thereafter, 4 ml of a mixed solution of DCM:TFA (trifluoroacetic acid):TIPS
20 (triisopropylsilane) = 94:1:5 was added and the mixture was stirred for 2
minute, and the solvent was removed by a nitrogen gas. This operation was
repeated five times, and suction filtration was conducted. The residue was
washed with DCM, triethylamine and DCM, then dried under reduced
pressure.

25 ii) Modification by methoxy type organic EL-dye

To the peptide resin dried under reduced pressure, 6 mL of 1-methyl-2-pyrrolidone (NMP) was added, and the mixture was stirred for 30 minute to swell, and 0.15 ml of triethylamine was added and the mixture was stirred. Further, 0.2 g of the active ester (6) was added and the mixture was
5 stirred at room temperature for 24 hours. Then, suction filtration was conducted, and the residue was washed with NMP and DCM and dried under reduced pressure.

iii) Cleavage from resin and deprotection of side chain

To the peptide resin dried under reduced pressure, 0.08 ml of m-cresol,
10 0.48 ml of thioanisole and 3.44 ml of TFA were added, and the mixture was stirred at room temperature for 1 hour. Then, the mixture was suction-filtrated and washed with TFA. TFA was distilled off under reduced pressure, and in an ice bath, 15 ml of ether was added. After treatment by ultrasound, the mixture was left for a while, and the supernatant was removed.
15 Then, in an ice bath, 15 ml of ethyl acetate was added. After ultrasound treatment, the mixture was left for a while. Then, the mixture was filtrated under reduced pressure and washed with ether, and dried under reduced pressure.

Orange color solid was obtained, and the yield was 0.29 g. HPLC
20 prpfiles before purification and after purification of the product are shown in Fig. 6A and Fig. 6B, respectively. A sample showing a peak around R.T. = 12.5 min was subjected to TOF-Mass measurement. As a result, a peak at 2057.33 was observed corresponding to a molecular weight of a complex of an EL-dye and peptide (EL-Peptide) of 2055.30, confirming the production of the
25 intended material (Matrix: α -cyano-4-hydroxycinnamic acid (α -CHCA); Fig. 7)

2. Detection of peptide

In the same manner as that of Example 1, a labeled peptide spotted on a glass substrate was detected. As the detection instrument, BIO·RAD molecule imager FX Pro was used. The laser wavelength was 488 nm and the scan interval was 50 nm.

(Result)

Fig. 8 shows an emission pattern of the labeled peptide, and (a), (b), (c), (d) and (e) show emission patterns of 10 fmol, 5 fmol, 1 fmol, 0.5 fmol and 0.1 fmol, respectively. The detection limit of the relative concentration of the labeled peptide was 0.1 fmol (100 amol), and therefore, detection in high sensitivity was possible. The reaction of the peptide and EL-dye was almost quantitative.

Example 4

<Labeling of proteins with dye, and detection thereof>

1. Labeling of protein with dye

An amino group of a lysine residue of bovine serum albumin (BSA) and an active ester of an organic EL-dye were reacted to form an amide bond for labeling of BSA. Specifically, to 58 μ l of carbonate buffer (pH 9.0) containing 4.0 mg (58 nmol) of BSA was added 40 μ l of a DMSO solution containing 3.6 mg (8.6 μ mol) of an active ester of an organic EL-dye (EL-OSu) and the mixture was shaken at 37°C for 24 hours. 0.1 M TEAA buffer (pH 7.0) was added so as to give the total volume of 1 ml, and components derived from BSA were separated using NAP-10 column (Pharmacia Sephadex G-25), and the separated solution was freeze-dried over night.

Identification of BSA labeled with an organic EL-dye was conducted by MALDI TOF MS. As shown in Fig. 9, the labeled BSA (Fig. 9B) had molecular weight increased by about 2200 as compared with the raw material (Fig. 9A), showing that about five organic EL-dyes was bonded thereto.

5 2. Detection of protein

(Result)

The prepared BSA emitted fluorescence in solid state as shown in Fig. 10. Thus, it was clarified that a protein can be labeled by an active ester of an organic EL-dye.